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Expression of a Bovine Growth Hormone Transgene Inhibits Pregnant Mare's Serum Gonadotropin-Induced Follicle Maturation in Prepuberal Gilts[1,2]

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ABSTRACT: Prepuberal gilts were injected with PMSG to determine whether expression of a bovine growth hormone (bGH) transgene inhibited preovulatory maturation of ovarian follicles. Seven transgenic (TG) gilts of line 3706, which expresses a mouse metallothionein-bGH transgene, and eight nontransgenic, control (C) gilts (128 to 147 d old) were injected with PMSG, 12.5 IU/kg BW, 72 h before necropsy. Surface ovarian follicles ≥ 1 mm in diameter were counted, measured for diameter, and aspirated for fluid. Follicles were classified morphologically as healthy or atretic and those with follicular fluid estradiol-17$\beta \geq 100$ ng/mL were classified as estrogen-active (EA). The number of follicles per gilt was 64.3 $\pm$ 6.1 (mean $\pm$ SEM) and did not differ significantly between bGH-TG and C gilts. The PMSG treatment induced growth of large (> 5 mm) follicles in both bGH-TG and C gilts. However, compared with C gilts, bGH-TG gilts had fewer ($P < .05$) large follicles (5.9 $\pm$ 1.5 vs 18.3 $\pm$ 5.4), a lower proportion of EA large follicles (35 $\pm$ 12.5 vs 69 $\pm$ 13.2%), and in large follicles less ($P < .05$) estradiol-17β (86 $\pm$ 17 vs 350 $\pm$ 69 ng/mL) and androstenedione (300 $\pm$ 33 vs 1,283 $\pm$ 221 ng/mL). Follicular fluid progesterone and inhibin did not differ significantly between bGH-TG and C gilts. The incidence of atresia among small and medium follicles did not differ significantly between bGH-TG and C gilts. These results indicate that chronic, unregulated expression of a bGH transgene in prepuberal gilts inhibited follicle growth in response to administration of PMSG and may have blocked estradiol-17β production in vivo by inhibiting theca interna androgen production.

Key Words: Gene Expression, Follicles, Androstenedione, Estradiol, Inhibin, Pigs

Introduction

Expression of bovine (**b**) growth hormone (**GH**) in transgenic (**TG**) pigs increased ADG, increased gain to feed ratio, and reduced the proportion of fat in carcasses of boars and gilts compared with nonexpressing littermates (Pursel et al., 1989). However, fertility in this animal model is limited because gilts are anestrous and boars have decreased libido (Pursel et al., 1989). The physiological processes that are

responsible for reduced fertility in bGH-TG swine have not been investigated.

Studies of reproductive processes in gilts administered pGH have provided some clues to the causes of infertility in bGH-TG gilts. Reproductive defects included delayed puberty (Bryan et al., 1989), reduced steroidogenic response of cultured ovarian granulosa cells to FSH (Bryan et al., 1989), delayed estrus after puberty (Kirkwood et al., 1988a), impaired ovulatory response to hCG (Kirkwood et al., 1988b), and attenuated estradiol-induced LH release in vivo (Kirkwood et al., 1988b). These reproductive problems may be related to impaired preovulatory follicle maturation, attenuated neuroendocrine signals from the central nervous system, and(or) impaired response to these signals by the anterior pituitary. The hypothesis tested in this experiment was that high levels of expression of a bGH transgene in prepuberal gilts would interfere with PMSG-induced preovulatory follicle maturation.

Materials and Methods

Animals

Gilts were from litters of nontransgenic gilts artificially inseminated with semen from a descendent of founder bGH-TG boar 3706, which integrated a fusion gene composed of a mouse metallothionein (**MT**) promoter and a bGH structural gene (Pursel et al., 1989). Presence of the MT-bGH gene in a pig's genome was detected by hybridization of dot blots of DNA from tail biopsies taken shortly after birth (Hammer et al., 1985). Expression of bGH was determined by RIA of plasma samples collected shortly after birth and during sexual maturation using a bGH RIA that did not measure porcine (**p**) GH (Miller et al., 1989). Gilts were weaned at 4 to 8 wk of age and were cared for as previously described (Pursel et al., 1989).

Seven bGH-TG gilts and eight non-TG sibling control (**C**) gilts (128 to 147 d old) were injected i.m. with PMSG (Dynasynt, Chicago, IL), 12.5 IU/kg BW, 72 h before necropsy. Euthanasia was by electrical shock and exsanguination. Blood was collected by venipuncture immediately before injection and at euthanasia. Plasma was stored at −20°C until it was assayed for bGH (Miller et al., 1989). Plasma bGH (mean ± SEM) was 229 ± 67 ng/mL in bGH-TG gilts and was nondetectable in C gilts at necropsy.

At necropsy, ovaries were excised and taken to the laboratory in ice-cold .9% saline solution. Follicles were counted, measured for diameter, and classified as small (1 to 2 mm), medium (3 to 5 mm), or large (> 5 mm). Follicular fluid was aspirated from each follicle with a finely drawn glass Pasteur pipette and classified as morphologically healthy or atretic when follicular fluid was clear or "milky" in appearance, respectively (Dailey et al., 1975; Guthrie et al., 1993). Follicular fluid from each medium and large follicle was pipetted into a 1.5-mL microfuge tube. Granulosa cells were pelleted by centrifugation at $1,200 \times g$ for 10 min and follicular fluid was stored at −20°C. Follicles were classified as estrogen-active (**EA**) with ≥ 100 ng of estradiol-17β/mL of fluid and estrogen-inactive with < 100 ng/mL (Guthrie et al., 1993).

Radioimmunoassay of Follicular Fluid Steroid Hormones

Aliquots of fluid from each follicle > 2 mm in diameter were diluted 100-fold with tissue culture medium 199. Estradiol-17β, progesterone, and androstenedione were quantified in duplicate in 10 or 100 μL of diluted fluid without solvent extraction using double antibody ^{125}I ligand RIA kits (Pantex, Santa Monica, CA) used previously in our laboratory (Guthrie et al., 1992). The assays were performed according to the instructions from the manufacturer. The minimum detectable amounts of estradiol-17β,

progesterone, and androstenedione were 1, 6, and 6 pg/tube, respectively. The mean CV of samples duplicated within assays was 11, 6, and 4% for estradiol-17β, progesterone, and androstenedione, respectively. The interassay CV was 10, 12, and 10% for estradiol-17β, progesterone, and androstenedione, respectively.

Radioimmunoassay of Follicular Fluid Inhibin

Inhibin was quantified in duplicate using a double-antibody RIA (Peninsula Laboratories, Belmont, CA) used previously in our laboratory (Guthrie et al., 1992). The antiserum (catalog #RAS7280) was generated in a goat to the synthesized 32 amino acid N-terminus portion of porcine inhibin α subunit conjugated to BSA. The inhibin α subunit fragment (Iα_{1-32}) was also used as the assay standard and was the ^{125}I ligand (catalog #Y7280) in the RIA. The assay was performed according to the instructions from the manufacturer. Dose titrations of porcine and bovine follicular fluid and partially purified bovine inhibin were parallel to serial dilutions of Iα_{1-32} (Bolt and Caldwell, 1992; Guthrie et al., 1992). The minimum detectable inhibin was 8 pg/tube. The mean CV for samples duplicated within assays and the interassay CV were 6.8 and 15.4%, respectively.

Statistical Analyses

All calculations were done with release 6.03 of SAS for the personal computer (SAS, 1987). The total number of follicles and percentage of large follicles per gilt were analyzed with PROC GLM using a one-way treatment structure for expression group, C vs bGH-TG. The number of follicles, percentage of healthy follicles, and percentage of EA follicles per gilt were analyzed with PROC GLM using a split-plot experimental design with expression group as the whole plot treatment and follicle size class as the subplot treatment. Expression group means within follicle size class were compared by using the CONTRAST statement in PROC GLM.

Two control gilts did not have atretic medium follicles and one bGH-TG gilt had no large follicles. Most of the large follicles were morphologically healthy. Therefore, two separate analyses were performed for each hormone, one for the 14 gilts that had healthy medium and large follicles and one for the 13 gilts that had both healthy and atretic medium follicles. In the first analysis, average values for each gilt of follicular fluid estradiol-17β, progesterone, androstenedione, and inhibin in healthy medium and large follicles were analyzed with PROC GLM using the split-plot experimental design described above, except for the absence of the small follicle size class. In the second analysis, average values for each gilt of follicular fluid hormones in healthy and atretic medium follicles were analyzed with PROC GLM

using a split-plot experimental design with expression group as the whole plot treatment and follicle health, healthy vs atretic, as the subplot treatment.

Results

The total number of surface follicles ≥ 1 mm in diameter per gilt did not differ for bGH-TG and C gilts (66.4 ± 8.3 vs 62.5 ± 8.9, respectively; mean $\pm$ SEM). The number of follicles per gilt was affected by follicle size class ($P < .01$), expression group ($P < .05$), and follicle size class $\times$ expression group interaction ($P \leq .05$; Table 1). The number of large and medium follicles were three- and twofold greater in C than in bGH-TG gilts. The number of small follicles was less in C than in bGH-TG gilts. The size (diameter in millimeters) of large follicles in bGH-TG and C gilts ($7.2 \pm .3$ vs $7.6 \pm .2$; mean $\pm$ SEM) did not differ significantly. The percentage of EA follicles was affected by expression group ($P < .05$) and follicle size class ($P < .05$). The percentage of EA was greater in C than in bGH-TG gilts and was greater in large than in medium follicles (Table 1). The percentage of morphologically healthy follicles was affected only by follicle size class ($P < .01$; it was 0% in small follicles, approximately 56% in medium follicles, and almost 100% in large follicles (Table 1).

Follicular fluid steroids and inhibin concentrations from healthy follicles are shown in Table 2. Estradiol-17β and androstenedione concentrations were affected by expression group ($P < .01$), follicle size class ($P < .01$), and the expression group $\times$ follicle size class interaction ($P < .05$). Estradiol-17β and androstenedione concentrations were greater in C than in bGH-TG gilt and greater in large than in medium follicles. Furthermore, the difference between medium and large follicles for estradiol-17β and androstenedione concentrations was greater in C than in bGH-TG gilts. Follicular fluid progesterone concentration was affected only by follicle size class ($P \leq .01$); progesterone

concentration was greater in large than in medium follicles. Follicular fluid inhibin concentration was affected by follicle size class ($P \leq .01$) and the interaction of expression group $\times$ follicle size class ($P < .01$). Overall, inhibin concentration was greater in large than in medium follicles, but the difference between large and medium follicles was greater in C than in bGH-TG gilts.

Analysis of follicular fluid from healthy and atretic medium follicles indicated that expression group was not a significant source of variation (Table 3). Estradiol-17β and inhibin immunoactivity were greater ($P \leq .01$) and progesterone was less ($P \leq .01$) in healthy than in atretic follicles. Androstenedione did not differ significantly between healthy and atretic follicles.

Discussion

In this experiment we found that the ovarian response to PMSG as indicated by decreased growth of large follicles and decreased follicular estradiol-17β production in vivo was impaired in bGH-TG compared with C gilts. The theca interna and granulosa cells of the pig possess aromatase activity, but granulosa cells lack 17α-hydroxylase-17,20-lyase activity and therefore require the theca interna contribution of aromatizable androgen to produce estradiol-17β (Ainsworth et al., 1990). Our results were similar to those of a study that evaluated the effects of a 30-d pGH treatment on the ovarian response to PMSG in prepuberal gilts (Bryan et al., 1990). In both studies, the PMSG-induced increase in follicular fluid estradiol-17β and androstenedione was blocked or attenuated in bGH-TG or pGH-treated gilts but did not affect progesterone concentration. Treatment with pGH alone also decreased follicular androstenedione (Bryan et al., 1990), but it had no effect on follicular fluid estradiol-17β (Bryan et al., 1989, 1990) or progesterone (Bryan et al., 1989). Results from gilts

Table 1. Distribution of healthy and estrogen-active follicles (mean $\pm$ SEM) in eight control and seven bovine growth hormone-transgenic (bGH-TG) gilts

Expression group	Follicle size class	No. of follicles[a]	Percentage healthy[b]	Percentage estrogen-active[c]
Control	Small	32.1 ± 3.6	0	ND[d]
bGH-TG		52.0 ± 7.6	0	ND
Control	Medium	12.1 ± 2.3	49.5 ± 10.3	11.0 ± 2.9
bGH-TG		8.6 ± 2.1	64.9 ± 8.5	5.9 ± 2.7
Control	Large	18.3 ± 5.4	$99.7 \pm .3$	68.9 ± 13.2
bGH-TG		5.9 ± 1.5	97.9 ± 2.1	35.1 ± 12.5

[a]Follicle size class ($P \leq .01$) and the size class by expression group interaction ($P \leq .05$) were significant sources of variation.
[b]Follicle size class ($P \leq .01$) was a significant source of variation.
[c]Follicle size class ($P \leq .05$) and expression group ($P \leq .05$) were significant sources of variation.
[d]Not determined.

Table 2. Steroid and inhibin concentrations (mean ± SEM) in fluid of healthy medium and large follicles from control and bovine growth hormone-transgenic (bGH-TG) gilts

Expression group	Follicle size class	No. of gilts	Estradiol-17β, ng/mL[a]	Androstenedione, ng/mL[a]	Progesterone, ng/mL[b]	Inhibin, ng/mL[c]
Control	Medium	8	47 ± 14	419 ± 163	100 ± 61	268 ± 44
	Large		350 ± 69	1,283 ± 221	117 ± 46	644 ± 64
bGH-TG	Medium	6	35 ± 9	121 ± 24	59 ± 18	359 ± 60
	Large		86 ± 17	300 ± 33	98 ± 18	497 ± 46

[a]Expression group ($P < .01$), follicle size class ($P < .01$), and their interaction ($P < .05$) were significant sources of variation.
[b]Follicle size class ($P < .01$) was a significant source of variation.
[c]Follicle size class ($P < .01$), and its interaction with expression group ($P < .01$) were significant sources of variation.

treated with bGH-TG or pGH indicate that follicular estradiol-17β was low because theca interna androgen production was inhibited. Treatment of prepuberal gilts with PMSG, containing both LH- and FSH-like biological activity (Guthrie et al., 1990), activates steroidogenesis in both theca interna and granulosa cells of maturing follicles (Evans et al., 1981; Tsang et al., 1985). We suggest that chronic high levels of GH, either from injection or expression of the bGH transgene, attenuated or blocked PMSG-induced theca interna 17α-hydroxylase-17,20-lyase activity.

The high incidence of follicular atresia among nonovulatory follicles during PMSG-induced preovulatory maturation was in agreement with the incidence of atresia in sexually mature pigs (Dailey et al., 1975; Guthrie et al., 1993) and with decreased numbers of follicles in PMSG-treated prepuberal gilts (Guthrie et al., 1990). We found no evidence that the atretic process differed in bGH-TG and C gilts.

Stimulatory actions of GH on the ovary have been reported and may be mediated directly (Adashi et al., 1985; Hammond et al., 1988) or indirectly through increased ovarian (Davoren and Hsueh, 1986; Hammond et al., 1988) or hepatic production of IGF-I (Sara and Hall, 1990). Results of numerous experiments indicate clearly that ovarian follicular cells secrete and respond to IGF-I and other growth factors (Hammond et al., 1988). However, the function of cultured granulosa cells may be different from their function in vivo, where they are exposed to factors from other ovarian cell types and circulating factors

from hepatic or hypophyseal origin. Effects of exogenous GH also may vary by species or physiological status; exogenous GH and gonadotropin work synergistically to induce preovulatory maturation in hypogonadotropic women (Blumenfeld, 1991) but not in normal women (Tapanainen et al., 1992).

The mechanisms by which GH attenuated PMSG-induced ovarian function in the pig are unknown. Results of recent studies indicate that IGF binding proteins may exert an inhibitory influence on gonadotropin action (Ui et al., 1989; Bicsak et al., 1991; Hammond et al., 1991). In addition to antagonism of PSMG-induced follicular development at the ovarian level, high concentrations of GH may act at the hypothalamic-hypophyseal level. Compared with its secretion in control gilts, FSH secretion in treated gilts was not affected by pGH treatment (Bryan et al., 1989) and did not alter the development of ovarian negative feedback on FSH secretion during sexual maturation in bGH-TG gilts (Guthrie et al., 1991). However, plasma LH was reduced in bGH-expressing gilts and boars of line 3607 during sexual maturation compared with nonexpressing control pigs (Guthrie et al., 1991). In contrast, injection of pGH had no significant effect on mean circulating concentrations of LH in pigs (Kirkwood et al., 1988b, 1989; Gilbertson et al., 1991); however, the pulsatile mode of LH release seemed to be slightly attenuated (Gilbertson et al., 1991). Therefore, the theca interna and perhaps the granulosa cells of follicles in bGH-TG or pGH-treated gilts could be less mature and require more time to respond to an injection of PMSG.

Table 3. Steroid and inhibin concentrations (mean ± SEM) in fluid of healthy and atretic medium follicles from control and bovine growth hormone-transgenic (bGH-TG) gilts

Expression group	Follicle health class	No. of gilts	Estradiol-17β, ng/mL[a]	Androstenedione, ng/mL	Progesterone, ng/mL[a]	Inhibin, ng/mL[a]
Control	Healthy	7	47 ± 6	419 ± 163	100 ± 61	268 ± 44
	Atretic		24 ± 14	412 ± 149	144 ± 71	162 ± 17
bGH-TG	Healthy	6	35 ± 9	121 ± 24	59 ± 18	359 ± 60
	Atretic		2 ± 2	41 ± 10	110 ± 35	124 ± 7

[a]Health class ($P \leq .01$) was a significant source of variation.

Other metabolic hormones such as insulin play a supporting role in maturation of ovulatory follicles (Booth, 1990). The ability of the GH/IGF system to antagonize the action of insulin as a regulator of cellular intermediary metabolism (Holly et al., 1988) may deprive ovarian follicles of metabolic substrates required for cell growth and steroidogenesis. The high circulating concentrations of insulin and glucose in pigs of the 3607 line indicate that they are insulin resistant (Guthrie et al., 1991) and may be another explanation for the ability of GH to interfere with PMSG-induced preovulatory maturation in the pig.

Implications

These results indicate that chronic, unregulated expression of a bovine growth hormone transgene in prepuberal gilts inhibited follicle growth in response to administration of pregnant mare's serum gonadotropin. The expression of the transgene may have reduced estradiol-17β production in vivo by inhibiting theca interna androgen production.

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